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$[Cp^*Rh(bpy)(H_2O)]^{2+}$ as a coenzyme substitute in enzymatic oxidations catalyzed by Baeyer–Villiger monooxygenases

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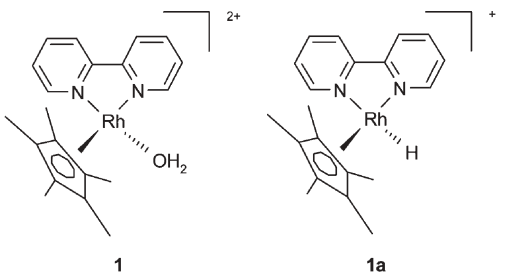
$[Cp^*Rh(bpy)(H_2O)]^{2+}$ was applied as a flavin regenerating reagent in BVMO catalyzed oxidations of organic sulfides to chiral sulfoxides.

In organic synthesis, the application of isolated enzymes depending on expensive coenzymes [*e.g.* $NADP^+$] is often hindered by the consumption of the respective coenzymes in stoichiometric amounts. The most likely solution to avoid this drawback is the coupling of the main reaction with an ancillary (enzymatic) reaction able to recycle the coenzyme.

Recently, it has been reported that certain organorhodium complexes can reduce some biologically active structures, such as flavins,[1] porphyrins[1] and nicotinamides.[1,2] Treatment of $[Cp^*Rh(bpy)(H_2O)]^{2+}$, **1** (Scheme 1)[3] with a formate salt leads to $[Cp^*Rh(bpy)H]^+$, **1a**. This compound (**1a**) is able to act as an electron donor capable of reducing the FAD cofactor. By this, the need for NADPH can be avoided. This methodology has been employed in enzymatic epoxidations catalyzed by an FAD-dependent styrene monooxygenase.[4] Another attractive feature is that, also, a cost-effective electrochemical method can be used for the regeneration of **1a**.[2,3]

In the present report, we describe the application of **1** in the enzymatic oxidation of organic sulfides and sulfoxides catalyzed by four isolated flavin-monooxygenases, namely: phenylacetone monooxygenase (PAMO)[5] from *Thermobifida fusca*, 4-hydroxyacetophenone monooxygenase (HAPMO)[6] from *Pseudomonas fluorescens* ACB, cyclohexanone monooxygenase (CHMO)[7] from *Acinetobacter* NCIMB 9871 and ethionamide monooxygenase (EtaA)[8] from *Mycobacterium tuberculosis*. These enzymes, which have been classified as Baeyer–Villiger monooxygenases (BVMOs, flavin containing enzymes),[9] were chosen on the basis of their sequence distance in order to cover an ample volume of sequence space for this class of enzymes[9] and thus maximize possible differences in their catalytic properties. These enzymes are able to catalyze the nucleophilic oxidation of ketones (Baeyer–Villiger reaction) as well as the electrophilic oxidation of various heteroatoms such as that of organic sulfides to the corresponding optical active sulfoxides,[10] valuable compounds for medicinal and fine chemistry.[11]



Scheme 1 Structures of organorhodium complexes involved in the flavin regeneration system.

Before testing the performance of **1** as a regenerating agent, $NADP^+$ and the glucose-6-phosphate/glucose-6-phosphate dehydrogenase recycling system were employed and both the conversion and enantiomeric excess so obtained were used as benchmarks for the model substrates tested. Using the enzymatic regeneration system, at first, enzymatic sulfoxidation was performed with PAMO and benzyl ethyl sulfide, **2**. It was found that **2** was oxidized to (*S*)-benzyl ethyl sulfoxide [(*S*)-**3**] with high enantiomeric excess (ee 98%, Table 1, entry 1). Next, **1**, synthesized according to the literature,[3] was employed at two concentrations (1.0 and 10.0 mM; Table 1, entries 2 and 3), in the presence of sodium formate, in the PAMO oxidation of **2**. In these conditions, both conversion and, even more so, enantiomeric excess were low but measurable. This indicates that the rhodium-based regeneration system is able to replace coenzyme regeneration.†

A schematic representation of the reactions that were likely involved is reported in Scheme 2, part a. The oxidation of the sulfide to the corresponding sulfoxide is obtained at the expense of formate ion and oxygen without the involvement of NADPH; in this case, **1a** directly reduces the FAD bound to the enzyme to

Table 1 PAMO oxidation of benzyl ethyl sulfide, **2**, and benzyl ethyl sulfoxide, (±)-**3**, using different recycling systems[a]

Entry	Substrate[b]	[**1**][c]	[$NADP^+$][c]	Conversion (%)[d]	Ee (%)[e]
1[f]	**2**	—	0.26	36	⩾98
2	**2**	1.0	—	22[g]	7
3	**2**	10.0	—	17	9
4	**2**	10.0	0.26	21	97
5	**2**	1.0	1.3	27	97
6[f]	(±)-**3**	—	0.26	49	93
7	(±)-**3**	10.0	—	10	<5
8	(±)-**3**	10.0	0.26	14	6
9	(±)-**3**	1.0	1.3	24	22

[a] Reactions stopped after 6 h for **2** and 4 h for (±)-**3**. [b] 16 mmol L^{-1}. [c] Concentrations expressed in mmol L^{-1}. [d] Measured by HPLC using acetanilide as internal standard. [e] Determined by chiral HPLC. [f] Reactions carried on using the glucose-6-phosphate/glucose-6-phosphate dehydrogenase system. [g] Reaction stopped after 24 h.

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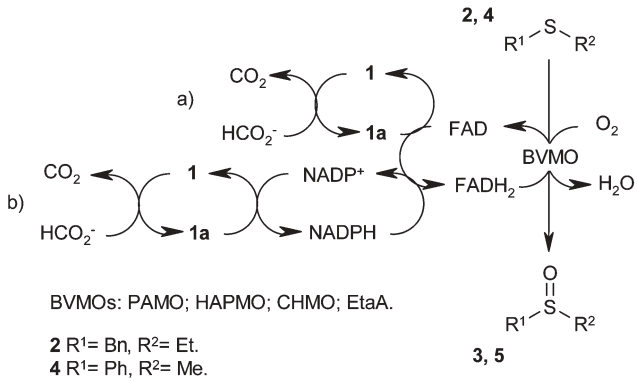

Scheme 2 Enzymatic oxidation of organic sulfides catalyzed by BVMOs using (a) complex **1**/formate and (b) adding $NADP^+$ to the mixture **1**/formate.

$FADH_2$. When the reaction was carried out in the presence of $NADP^+$ (0.26 mM) and **1** (Table 1, entry 4), PAMO selectivity was completely recovered, and (*S*)-**3** was obtained with almost the same enantiomeric excess as that of the control reaction, even though with lower conversion. In addition, when the concentration of **1** was decreased to 1.0 mM and that of $NADP^+$ was increased to 1.3 mM, a small improvement in the conversion was observed (Table 1, entry 5). In this case the scenario should be as depicted in Scheme 2, part b. Compound **1a** reduces $NADP^+$ to the enzymatically active NADPH isomer which, in turn, reduces FAD to $FADH_2$.[1,2]

Racemic benzyl ethyl sulfoxide, (±)-**3**, was also studied as a PAMO substrate. Using the enzymatic reaction as an NADPH recycling system, the (*R*)-**3** enantiomer was oxidized to the corresponding sulfone, leaving the remaining (*S*)-**3** with good enantiomeric excess (Table 1, entry 6). With **1**, the biocatalyst lost its selectivity, yielding racemic (±)-**3** as the remaining compound. Although the reactions performed with two different mixtures of $NADP^+$/ **1**/ formate showed improved enzyme selectivity, still very low enantiomeric excesses for the remaining substrate and low degrees of conversion were obtained (Table 1, entries 8 and 9).

Enzymatic oxidation of thioanisole (methyl phenyl sulfide, **4**) with HAPMO and enzymatic coenzyme recycling, leads to enantiopure (*S*)-thioanisole sulfoxide, (*S*)-**5**, with high yield (Table 2, entry 1).[10] When **1** was used at 1.0 and 10.0 mM concentrations the racemic sulfoxide was recovered in low yields (Table 2, entries 2 and 3). No significant improvement in the biocatalyst properties was found in the presence of $NADP^+$, when 10 mM **1** was utilized (Table 2, entries 4 and 5). Instead, at a lower concentration of **1** (1 mM), (*S*)-**5** was obtained with higher conversion and good optical purity (ee 81%) (Table 2, entry 7). Thioanisole sulfoxide **5** was also oxidized by HAPMO to the corresponding sulfone, but no selectivity was found for any of the recycling systems tested (Table 2, entries 8–11). However the combined use of **1** and $NADP^+$ gave a conversion close to that of the control reaction.

CHMO is the best known flavin containing monooxygenase for synthetic applications[10] and has been successfully employed in the selective oxidation of several sulfides.[12] The oxidation of **4** led to the enantiopure (*R*)-**5** with high conversion (Table 3, entry 1). The oxidation of **4** carried out in the presence of 10 mM of **1** yielded the sulfoxide **5** with low enantiomeric excess and conversion (Table 3, entry 2). The presence of $NADP^+$ increased both the conversion and the enantiomeric excess of (*R*)-**5**. A decrease in the concentration of **1** (1.0 mM), and an increase in that of $NADP^+$, induced a slight improvement in enantioselectivity and a higher degree of conversion (Table 3, entry 4). No reaction was observed when the oxidation of (±)-**5** to the corresponding sulfone was attempted.

Finally, EtaA was investigated. In the control reaction, **2** was oxidized to the corresponding *S*-sulfoxide with good selectivity (ee 83%) (Table 4, entry 1). When **1** was employed at 1.0 or 10.0 mM concentration, **3** was obtained in almost racemic form (Table 4, entries 2 and 3). The same result was observed using a low concentration of $NADP^+$ (Table 4, entry 4). Only when the concentration of **1** was reduced to 1.0 mM and that of $NADP^+$ was increased to 1.3 mM, was (*S*)-**2** formed with an enantiomeric excess of 28% and a conversion close to that achieved in the

Table 2 HAPMO oxidation of thioanisole, **4**, and methyl phenyl sulfoxide, (±)-**5**, using different recycling systems

Entry	Substrate[a]	[**1**][b]	[NADP][b]	*t* (h)	Conversion (%)[c]	Ee (%)[d]
1[e]	**4**	—	0.26	24	96	⩾98
2	**4**	1.0	—	48	4	<5
3	**4**	10.0	—	24	10	<5
4	**4**	10.0	0.26	24	11	6
5	**4**	10.0	1.3	24	18	11
6	**4**	5.0	0.72	24	21	46
7	**4**	1.0	1.3	24	29	81
8[e]	(±)-**5**	—	0.26	20	30	<5
9	(±)-**5**	1.0	—	40	14	<5
10	(±)-**5**	10.0	—	20	20	<5
11	(±)-**5**	10.0	0.26	20	29	<5

[a] 16 mmol L^{-1}. [b] Concentrations expressed in mmol L^{-1}. [c] Measured by HPLC using acetanilide as internal standard. [d] Determined by chiral HPLC. [e] Reactions carried on using the glucose-6-phosphate/glucose-6-phosphate dehydrogenase system.

Table 3 CHMO oxidation of thioanisole, **4**, using different recycling systems

Entry	Substrate[a]	[**1**][b]	[NADP][b]	Conversion (%)[c]	Ee (%)[d]
1[e]	**4**	—	0.26	88	⩾98
2	**4**	10.0	—	5	9
3	**4**	10.0	0.26	31	91
4	**4**	1.0	1.3	55	94

[a] 16 mmol L^{-1}. [b] Concentrations expressed in mmol L^{-1}. [c] After 24 h and measured by HPLC using acetanilide as internal standard. [d] Determined by chiral HPLC. [e] Reactions carried using the glucose-6-phosphate/glucose-6-phosphate dehydrogenase system.

Table 4 EtaA oxidation of benzyl ethyl sulfide, **2**, using different recycling systems

Entry	Substrate[a]	[**1**][b]	[NADP][b]	*t* (h)	Conversion (%)[c]	Ee (%)[d]
1[e]	**2**	—	0.26	16	24	83
2	**2**	1.0	—	32	6	<5
3	**2**	10.0	—	16	10	<5
4	**2**	10.0	0.26	16	19	<5
5	**2**	1.0	1.3	16	26	28

[a] 16 mmol L^{-1}. [b] Concentrations expressed in mmol L^{-1}. [c] Measured by HPLC using acetanilide as internal standard. [d] Determined by chiral HPLC. [e] Reactions carried using the glucose-6-phosphate/glucose-6-phosphate dehydrogenase system.

control reaction. No oxidation was observed for (±)-**5** with any of the conditions tested.

On the whole, the results indicate that the enantioselectivity displayed by the four studied BVMOs in the oxidation of sulfides is very low, when using only **1** as a coenzyme substitute. Similar results (ee 26%) were previously obtained with another monooxygenase, styrene monooxygenase (StyA),[13] in the oxidation of **4** to **5**.[4] The lack of enantioselectivity might be ascribed to i) formation of hydrogen peroxide obtained through oxygen reduction[2] by **1** and consequent abiotic oxidation of the sulfide to the racemic sulfoxide or ii) spontaneous sulfide oxidation by air. However, these hypotheses were ruled out by the fact that in control experiments, conducted in the presence of the various reagents but without the monooxygenases, the formation of sulfoxides did not occur. Thus, sulfoxides are produced by genuine enzyme catalysis and, more importantly, **1** is able to reduce the FAD associated with the enzymes.

Although styrene monooxygenase and BVMOs are closely related enzymes, there are differences in the role of FAD. In styrene monooxygenase the cofactor is loosely bound to the enzyme and can diffuse in the medium,[13] whereas in the studied BVMOs the flavin cofactor is tightly bound to the enzyme, which implies that **1a** has to enter the enzyme active site in order to reduce the cofactor. This latter hypothesis is also supported by the finding that it is possible to accommodate **1a**, by manual docking, in the active site of PAMO (data not shown). Because of the bulkiness of **1a** compared to that of NADPH, we must also assume a certain degree of enzyme flexibility to make it possible to accommodate **1a** in enzyme active site.

When $NADP^+$ is present in the medium, it is likely that the reaction mechanism is not as simple as illustrated in Scheme 2, part b. A more reliable representation might be that both **1a** and NADPH compete for the same active site. This view is also supported by literature kinetic data demonstrating that **1a** is more efficient in reducing $NADP^+$ to NADPH than FAD to $FADH_2$.[2] Therefore a decrease in concentration of **1** (*e.g.* from 10 to 1 mM) and an increase in that of $NADP^+$ (*e.g.* from 0 to 1.3 mM) will decrease the fraction of FAD directly reduced by **1a** (Scheme 2, part a) and increase that reduced through NADPH (Scheme 2, part b). This partial switch of mechanism was also reflected in the general increase of both conversion and enantiomeric excess. For example, with PAMO the recovery of the enantioselectivity was full when supplementing the system with $NADP^+$ (Table 1, entries 4 and 5). The different influence of **1a** and NADPH on enzyme enantioselectivity is quite intriguing. It has been shown previously that, in the catalytic cycle of BVMOs, NADPH is bound to the enzyme during the substrate oxidation step.[14] We hypothesize that, as $NADP^+$ is bound during the oxidation reaction, it is also part of the active site cavity, thereby tuning the (enantio)selectivity of the enzyme. Instead **1a**, even if able to reduce FAD, will not show any real affinity for the enzyme and will diffuse from the active site before oxidation takes place. Therefore, in this case, the enzyme active site will be less defined, resulting in lowered selectivity.

In conclusion, we have demonstrated that **1**/formate can be used as a coenzyme substitute in BVMO oxidations as **1a** is able to reduce the enzyme bound FAD. Unfortunately, the enantioselectivity displayed by the enzymes in these conditions was disappointingly low. The presence of $NADP^+$ in the reaction medium restored selectivity. This suggests that the role of NADPH in the catalytic process is not only that of a mere reducing agent: it enhances the enantioselectivity by properly shaping the active site of these enzymes.

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Notes and references

† General method for enzymatic oxidations using the rhodium complex as coenzyme: the organic sulfide or sulfoxide (1.9–2.7 mg; 16 mmol L^{-1}) was added to potassium phosphate buffer (50 mM, pH 9.0, 0.9 mL) containing sodium formate (6.8 mg; 0.1 M), an aqueous solution of **1** (100 mM, 0.1 mL), 1.0 U of the flavin containing monooxygenase and acetanilide (0.02 mg) as an internal standard. When necessary, different amounts of $NADP^+$ were added. The system was shaken at 250 rpm and 25 °C. The reaction was then stopped, worked up and analyzed by chiral HPLC (Chiracel OD, Daicel, chiral column; λ_{254} nm; flow rate 1 mL min^{-1}; eluent: light petroleum ether, *i*-PrOH).[12]

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